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Backbone Extension by methylene insertion as a Strategy to Restore Duplex Stability of Sulfonamide-Linked Oligodeoxynucleotides While Preserving RNase H Activity

Kohji Seio,* Hiroki Mikagi, Haruka Tanabe, Shigetoshi Tachibana, Yoshiaki Masaki and Eitaro Murakami

Department of Life Science and Technology, Institute of Science Tokyo

4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan.

seio.k.2552@m.isct.ac.jp

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General

Chemicals: Reagents were purchased from commercial suppliers (Fujifilm Wako Pure Chemicals, Tokyo Kasei Kogyo, Kanto Chemical, Sigma-Aldrich, Glen Research, Hongene Biotech, Eurofins DNA Synthesis, Link Technologies, and Ajinomoto Bio-Pharma Services), and used without further purification.

Kieselgel 60 F245 (Merck) was used for TLC analyses. Appropriate mixtures of *n*-hexane, ethyl acetate, dichloromethane, methanol, toluene, and acetonitrile was used as the development solvent. TLC spots were visualized by UV (254 nm), and staining reagents (5% sulfuric acid/methanol solution, *p*-anisaldehyde solution, iodine powder).

Spectrometry: NMR spectra were measured using a Varian AS500 (500 MHz for ^{1}H) or a JEOL JNM-ECZL400S (400 MHz for ^{1}H, 101 MHz for ^{13}C and 162 MHz for ^{31}P). DMSO-d6, $CDCl_3$, and CD_3CN were used as the solvents, and the peaks derived from each solvent were used as internal standards for ^{1}H and ^{13}C NMR The chemical shifts of the solvents were DMSO: 2.50 ppm, $CHCl_3$: 7.26 ppm, CH_3CN: 1.94 ppm for ^{1}H, and DMSO: 39.5 ppm, CDCl3: 77.0 ppm, CD3CN: 1.32 ppm for ^{13}C.

ESI-TOF-MS measurements were performed on a micrO TOF II (Bruker Daltonics), and MALDI-TOF-MS measurements were performed on a UltraleXtreme (Bruker Daltonics) at the Analysis Division, Open Facility Center, Tokyo Institute of Technology.

Automated oligonucleotide synthesis: Oligonucleotide synthesis was performed on nS-II8 automated oligonucleotide synthesizer from Gene Design, Inc. Natural-type deoxyphosphoramidite unit (dT phosphoramidite, dG^{ibu} phosphoramidite, dA^{Bz} phosphoramidite, dC^{Bz} phosphoramidite), solid-supports, activator, oxidant, Cap A solution (5% acetic anhydride/tetrahydrofuran solution), Cap B solution (10% 1-methylimidazole tetrahydrofuran-pyridine solution), and sulfurizing agent were purchased from Glen Research; 5-Me-dC^{Bz} phosphoramidite was purchased from Link technologies. Each LNA phosphoramidite unit (LNA-T phosphoramidite, LNA-A^{Bz} phosphoramidite, LNA-G^{dmf} phosphoramidite, LNA-5-Me-C^{Bz} phosphoramidite) was purchased from Hongene Biotech. Acetonitrile (super dehydrated) and deblocking solution (3 w/v% trichloroacetic acid/dichloromethane solution), [(N,N-dimethylaminomethylidene)amino]-3H-1,2,4-dithiazoline-3-thione (DDTT) for preparation of sulfurizing agents were purchased from Fujifilm Wako Pure Chemical Co. Ltd.

Modified phosphoramidite unit used and LNA phosphoramidite unit were dissolved in dry acetonitrile to 0.1 M after thorough azeotropic drying with dry pyridine, dry toluene and dry dichloromethane. LNA-5-methyl-C^{Bz} was also dissolved in dry acetonitrile/dry dichloromethane (1:1, v/v) to 0.1 M and applied to the automated DNA synthesizer. The activator was 0.25 M 5-benzylthio-1*H*-

tetrazole/acetonitrile. The sulfurizing agent was 0.05 M Sulfurizing Reagent II/pyridine/acetonitrile solution or DDTT dissolved in dry pyridine/dry acetonitrile (3:2, v/v) prepared under an argon atmosphere. Coupling times were 12 min for each modified nucleic acid and each LNA phosphoramidite unit and 6 min for natural-type DNA, and each coupling reaction was performed twice. The oxidation treatment time was repeated twice for 5 seconds each, and the sulfurization treatment time was repeated twice for 5 minutes each. The synthesized ODN and ASO were cleaved from the solid supports and deprotected at the base by treatment with 28% ammonia at 55 °C for 16 hours. Ammonia was then removed using a centrifugal evaporator, followed by purification using Sep-Pak C18 (Waters), and purification and purity confirmation using reverse phase HPLC.

Manual synthesis: For each ODN (**ODN2**–**9**), the incorporation of the modified dimers TnscT (XX) , whose phosphoramidite was synthesized according to ref13 in the main text, and TnsccT (YY) using **8** was carried out by transferring the solid supports (1 μmol), on which the sequences had been synthesized up to the preceding position using an automated DNA synthesizer, into a glass filter sealable with a three-way stopcock equipped with an argon balloon (hereinafter referred to as the solid-phase cylinder). First, deprotection of the 5′-terminal DMTr group on the solid-phase carrier was carried out using a 1% trifluoroacetic acid/dichloromethane solution, washed with dichloromethane and acetonitrile. In the coupling reaction, 20 equivalents of XX or YY were added to a solid-phase cylinder along with 40 equivalents of 1*H*-tetrazole, and dissolved in 500 μL of dehydrated acetonitrile. For **ODN6**, the coupling reaction was performed twice under identical conditions. The mixture was then shaken for 4 minutes and then washed with acetonitrile. Next, a solution of 0.1 M DMAP dissolved in dehydrated pyridine/anhydrous acetic acid (9:1, v/v) was added, and the resulting solution was shaken for 2 minutes. Finally, a 0.1% iodine solution dissolved in pyridine/water (1:9, v/v) was added as the oxidizing agent, and the mixture was shaken for 2 minutes to oxidize the phosphate. The mixture was then washed with acetonitrile and dichloromethane, and the oligonucleotide-loaded solid-phase carrier was loaded onto an empty column to continue automated synthesis.

For each ASO (**ODN2**, **3**), the incorporation of the modified dimers XX (synthesized according to previous reports) and YY (compound **8**) was carried out by transferring the solid supports (1 μmol), on which the sequences had been synthesized up to the preceding position using an automated DNA synthesizer, into a glass filter sealable with a three-way stopcock equipped with an argon balloon (hereinafter referred to as the solid-phase cylinder). First, deprotection of the 5′-terminal DMTr group on the solid-phase carrier was carried out using a 1% trifluoroacetic acid/dichloromethane solution, washed with dichloromethane and acetonitrile, and dried under vacuum for 5 min. In the coupling reaction, 20 equivalents of XX or YY and 40 equivalents of 1*H*-tetrazole were added to the solid-phase cylinder and dissolved in 500 μL of dehydrated acetonitrile. The mixture was then shaken under an argon atmosphere for 24 minutes and then washed with acetonitrile. Each coupling reaction was

performed twice under identical conditions. Next, DDTT was dissolved in dehydrated pyridine/dehydrated acetonitrile (3 : 2, v/v) to a concentration of 0.05 M as a sulfurizing agent, and sulfurized by shaking for 7 minutes. Then, after washing with acetonitrile, a solution of 0.1 M DMAP dissolved in dehydrated pyridine/acetic anhydride (9 : 1, v/v) was added, and the resulting solution was shaken for 2 or 3 minutes. Finally, after washing with acetonitrile and dichloromethane, the column was dried under vacuum for 5 or 10 min until sufficiently dry, and the oligonucleotide-loaded solid phase carrier was loaded onto an empty column.

Circular dichroism (CD) spectra of duplexes and dimers: We used a circular dichroism dispersometer J-1100 (Japan Spectroscopy).

ODN/RNA (1:1) duplexes were dissolved in phosphate buffer (10 mM sodium phosphate, 100 mM sodium chloride, 0.1 mM EDTA, pH 7.0) to a final concentration of 4.0 μM duplex. The duplexes were annealed by heating at 95 °C for 3 min, then slowly cooled to room temperature over 1 h. CD spectra were measured from 360 nm to 190 nm at 20 °C. The scanning speed was 100 nm min^{-1}, and the spectra were averaged over 10 scans. In the measurements of dimers, the dimers were dissolved in H_2O–DMSO (95 : 5, v/v). The concentration of the dimers was 120 μM. Then, CD spectra were measured using a circular dichroism dispersometer at varying temperatures of 10 °C, 20 ° C, 30 °C, 40 °C, 50 °C, and 60 °C. The measurement range was 350 nm–190 nm. The scanning speed was 200 nm min^{-1}, and the spectra were averaged over 8 or 10 scans.

Concentration of oligonucleotides: Concentrations of oligonucleotides were calculated by using the ε_{260} calculated on OligoAnalyzer provided by Integrated DNA Technologies assuming that the extinction coefficients of modified nucleosides were the same as that of unmodified ones.

ODN1-9

ε = 153000 L/(mole·cm)

ASO1-3

ε = 147000 L/(mole·cm)

HPLC analyses: Reversed-phase HPLC analyses were performed using an XBridge Shield RP18 column (4.6 × 150 mm, Waters) for oligonucleotides and a Hydrosphere C18 column (4.6 × 150 mm, YMC) for enzymatic digestion samples. For oligonucleotides, a linear gradient of 5–45% methanol was applied over 30 min at 60 °C using solvent A (a mixture of 8 mM triethylamine and 0.1 M 1,1,1,3,3,3-hexafluoro-2-propanol) and solvent B (methanol). For enzymatic digestion samples, a linear gradient of 0–30% acetonitrile was applied over 30 min at 20 °C using solvent C (0.1 M ammonium acetate) and solvent D (acetonitrile). The flow rate was 1.0 mL/min.

Preparative reversed-phase HPLC was performed using an XBridge BEH Shield C18 column (10 ×

250 mm, Waters). A linear gradient of 5–45% methanol was applied over 30 min at 65 °C using solvent A (a mixture of 8 mM triethylamine and 0.1 M 1,1,1,3,3,3-hexafluoro-2-propanol) and solvent B (methanol). The flow rate was 3.0 mL/min.

RNase H Cleavage Assay and Denaturing PAGE Analysis

Preparation of annealing solution (Solution A). Each ASO (1 μL of a 1 μM solution) was mixed with 1 μL of a 1 μM solution of a 5′-6FAM-labeled complementary RNA (3′-UGGCUCCGAACGUAUG-FAM-5′), 2.5 μL of annealing buffer (200 mM Tris-HCl, pH 8.0; 300 mM KCl), 0.5 μL of 1% (v/v) Tween 20, and 5 μL of nuclease-free water to a final volume of 10 μL (Solution A). The mixture was incubated at 65 °C for 2 min on a heat block and then allowed to cool to room temperature over 1 h.

Preparation of RNase H solution (Solution B). E. coli RNase H (3 μL of a 10 mU/μL solution, Takara Bio Inc.), 3 μL of 1% (v/v) Tween 20, 15 μL of 4× reaction buffer (200 mM Tris-HCl, pH 8.0; 300 mM KCl; 80 mM $MgCl_2$; 20 mM dithiothreitol [DTT]), and 39 μL of mQ were combined to a final volume of 60 μL.

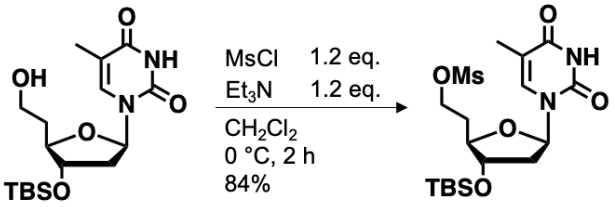
RNase H cleavage reaction.

Solution B (2.5 μL) was added to Solution A (10 μL), and the mixture was incubated at 37 °C for 10 min on a heat block. The reaction was quenched by adding 12.5 μL of denaturing loading buffer (10 M urea, 50 mM EDTA·2Na, 0.1w/v% bromophenol blue).

Denaturing polyacrylamide gel electrophoresis.

A denaturing gel was prepared as follows: 250 mL of 40% (w/v) acrylamide/bis-acrylamide solution (29:1 ratio), 50 mL of ; UltraPure™ 10× TBE buffer (1.0 M Tris, 0.9 M boric acid, 0.01 M EDTA; Invitrogen), and 210 g of urea were dissolved and brought to a final volume of 500 mL with mQ (final concentrations: 20% acrylamide and 7 M urea). To 40 mL of this gel solution, 200 μL of 10% (w/v) ammonium persulfate (APS) and 40 μL of N,N,N′,N′-tetramethylethylenediamine (TEMED) were added. After gentle mixing by inversion, the solution was poured into a gel cassette and allowed to polymerize overnight at room temperature. The gel was pre-run in 1× TBE buffer at 60 W and 60 °C until the temperature equilibrated and residual APS was removed. Each sample (4 μL) was then loaded and electrophoresed at 60 W and 60 °C for 40 min. FAM-labeled RNA species were detected using a fluorescence laser scanner (Typhoon FLA-9500; PMT voltage: 500 V, FAM channel), and the band intensities were quantified using ImageJ.

Compound 2

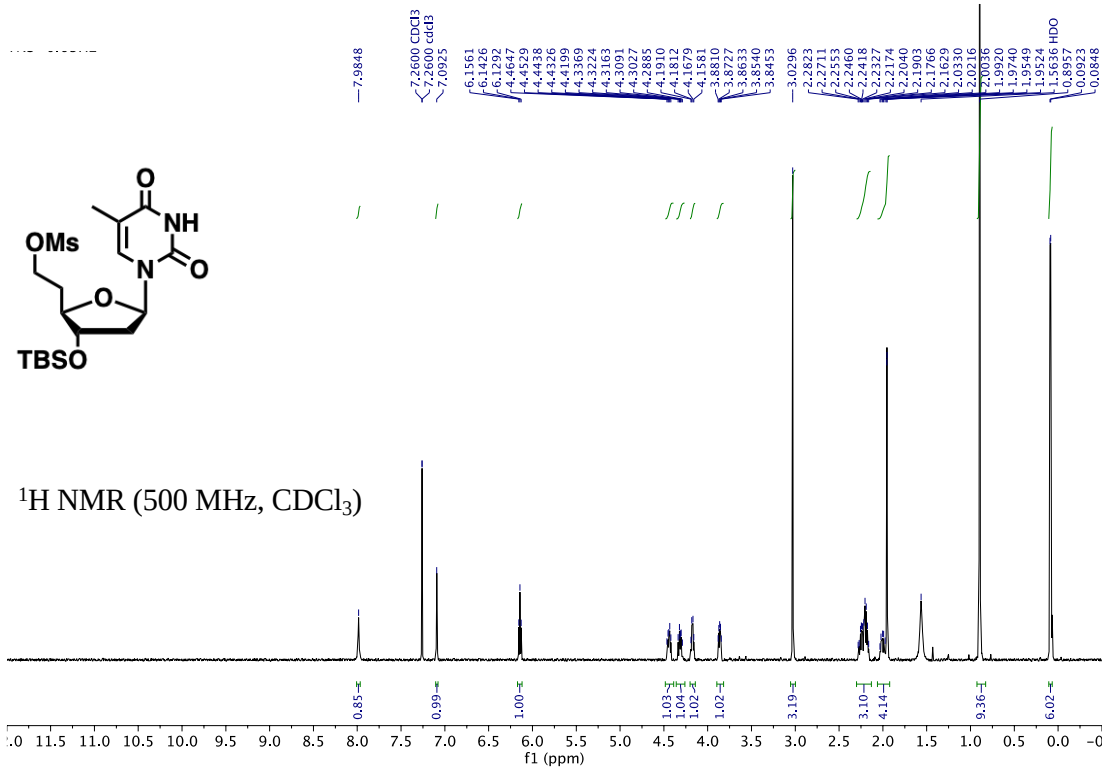


Compound **1** (443 mg, 1.2 mmol) was dissolved in CH_2Cl_2 (4.8 mL), and the solution was cooled to 0 °C. Then, Et_3N (204 µL, 1.4 mmol) and MsCl (114 µL, 1.4 mmol) were added. The resulting reaction mixture was stirred at 0 °C for 2 h. CH_2Cl_2 (35 mL) was added, and the organic layer was separated and washed twice with saturated $NaHCO_3$ solution (30 mL), and once with saturated NaCl solution (30 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated *in vacuo* to give crude, which was purified using flash chromatography on silica gel using a gradient from 10 to 80% EtOAc in *n*-hexane and gave the desired product **2** as colorless amorphous in 84% yield (450 mg, 1.00 mmol).

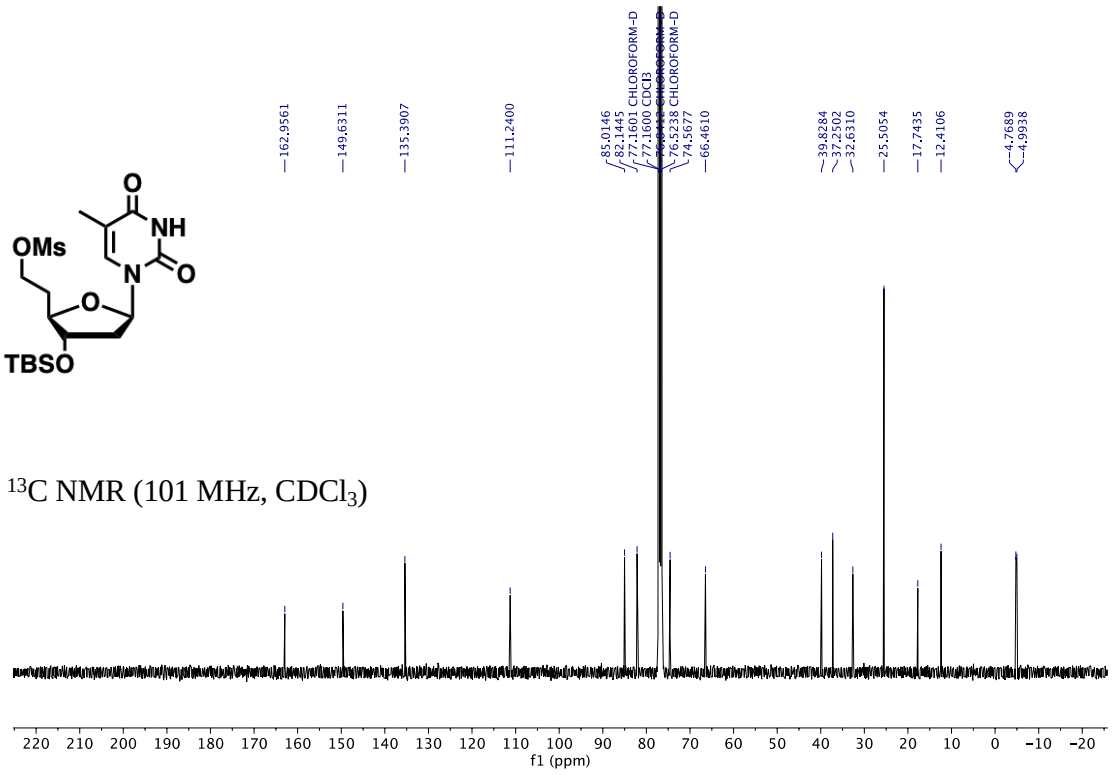
^{1}H NMR (500 MHz, $CDCl_3$) δ 7.98 (s, 1H), 7.09 (s, 1H), 6.14 (t, J = 6.7 Hz, 1H), 4.46-4.42 (m, 1H), 4.32-4.29 (m, 1H), 4.19-4.16 (m, 1H), 3.86 (dt, J = 9.0, 4.2 Hz, 1H), 3.03 (s, 3H), 2.28-2.16 (m, 3H), 2.03-1.95 (m, 4H), 0.90 (s, 9H), 0.09 (d, J = 3.8 Hz, 6H).

^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ 163.0, 149.6, 135.4, 111.2, 85.0, 82.1, 74.6, 66.5, 39.8, 37.3, 32.6, 25.5, 17.7, 12.4, -4.8, -5.0.

HRMS: m/z (ESI) calcd. for $C_{18}H_{32}N_2NaO_7SSi^+$ $[M + Na]^+$ 471.1592, found 471.1599.

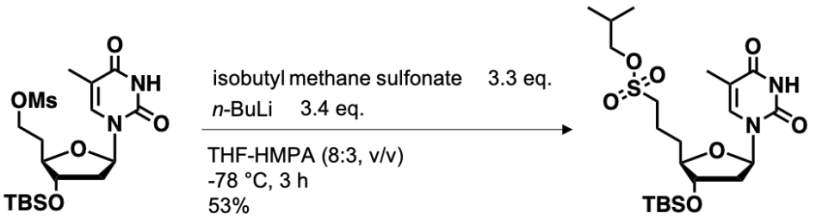


^{1}H NMR (500 MHz, $CDCl_3$)



^{13}C NMR (101 MHz, $CDCl_3$)

Compound 3

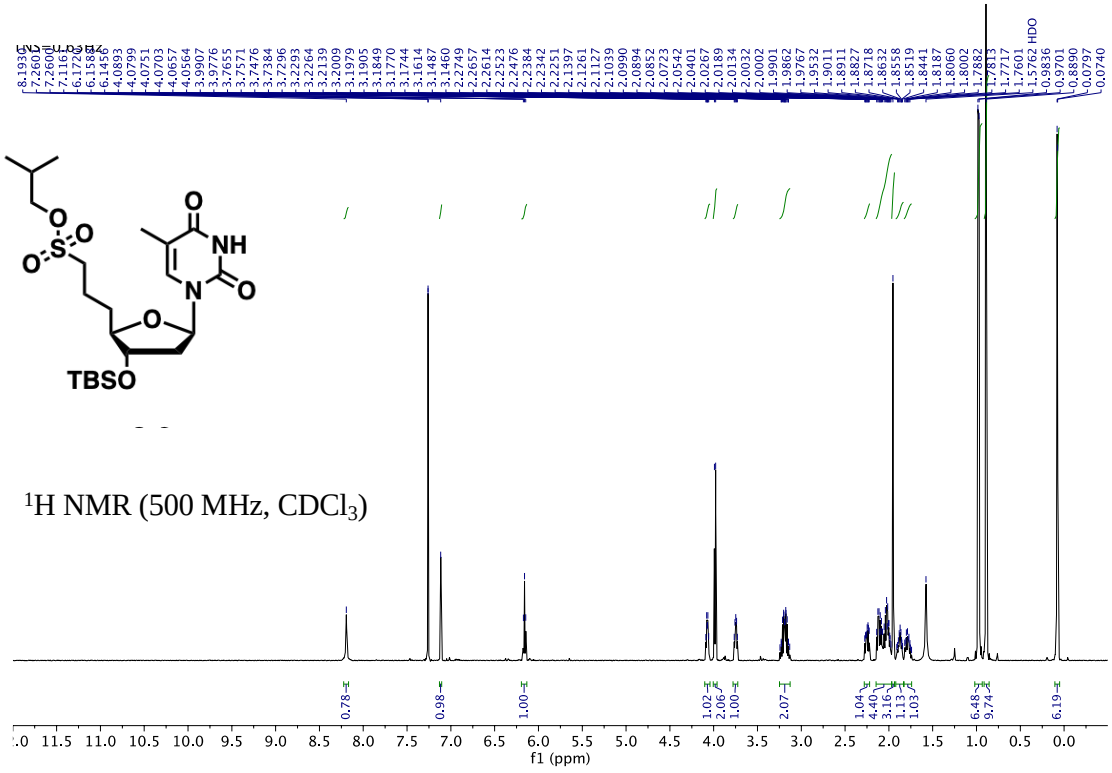


To a stirred solution of isobutyl methane sulfonate (203 µL, 1.5 mmol) in THF (500 µL) and HMPA (400 µL) *n*-BuLi (750 µL, 1.5 mmol) was added dropwise at -78 °C. The resulting reaction mixture was stirred at -78 °C for 3 h. To this reaction mixture compound **2** (198 mg, 0.4 mmol), which was rendered anhydrous by co-evaporation with dry toluene four times, was added dropwise in THF (500 µL) solution at -78 °C. The resulting reaction mixture was stirred at -78 °C for another 3 h. The solution was added dropwise to 0.1 M phosphate buffer (45 mL) at 0 °C, after which the mixture was diluted with EtOAc (150 mL). The solution was washed once with H_2O (70 mL) and twice with saturated NaCl solution (70 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated *in vacuo* to give crude, which was purified using flash chromatography on silica gel using a gradient from 10 to 80% EtOAc in *n*-hexane and gave the desired product **3** as colorless solid in 53% yield (117 mg, 0.23 mmol).

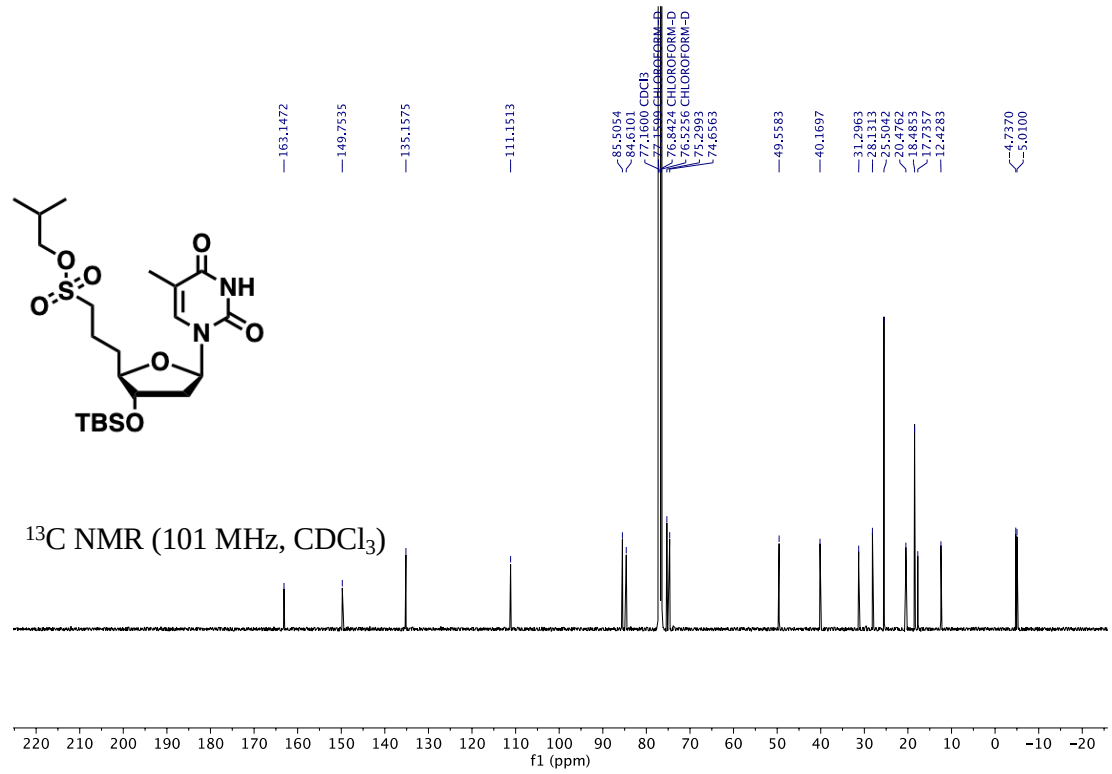
^{1}H NMR (500 MHz, $CDCl_3$) δ 8.19 (s, 1H), 7.12 (s, 1H), 6.16 (t, J = 6.6 Hz, 1H), 4.07 (dt, J = 6.9, 4.7 Hz, 1H), 3.98 (d, J = 6.5 Hz, 2H), 3.75 (dt, J = 9.0, 4.3 Hz, 1H), 3.24-3.13 (m, 2H), 2.27-2.23 (m, 1H), 2.14-1.98 (m, 4H), 1.95 (s, 3H), 1.91-1.84 (m, 1H), 1.82-1.74 (m, 1H), 0.98 (d, J = 6.8 Hz, 6H), 0.89 (s, 9H), 0.08 (d, J = 2.9 Hz, 6H).

^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ 163.1, 149.8, 135.2, 111.2, 85.5, 84.6, 75.3, 74.7, 49.6, 40.2, 31.3, 28.1, 25.5, 20.5, 18.5, 17.7, 12.4, -4.7, -5.0.

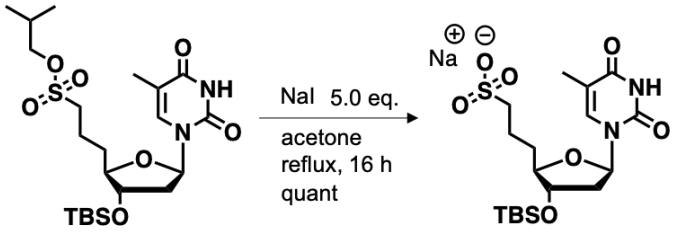
HRMS: m/z (ESI) calcd. for $C_{22}H_{40}N_2NaO_7SSi^+$ $[M + Na]^+$ 527.2218, found 527.2220.



^{1}H NMR (500 MHz, $CDCl_3$)



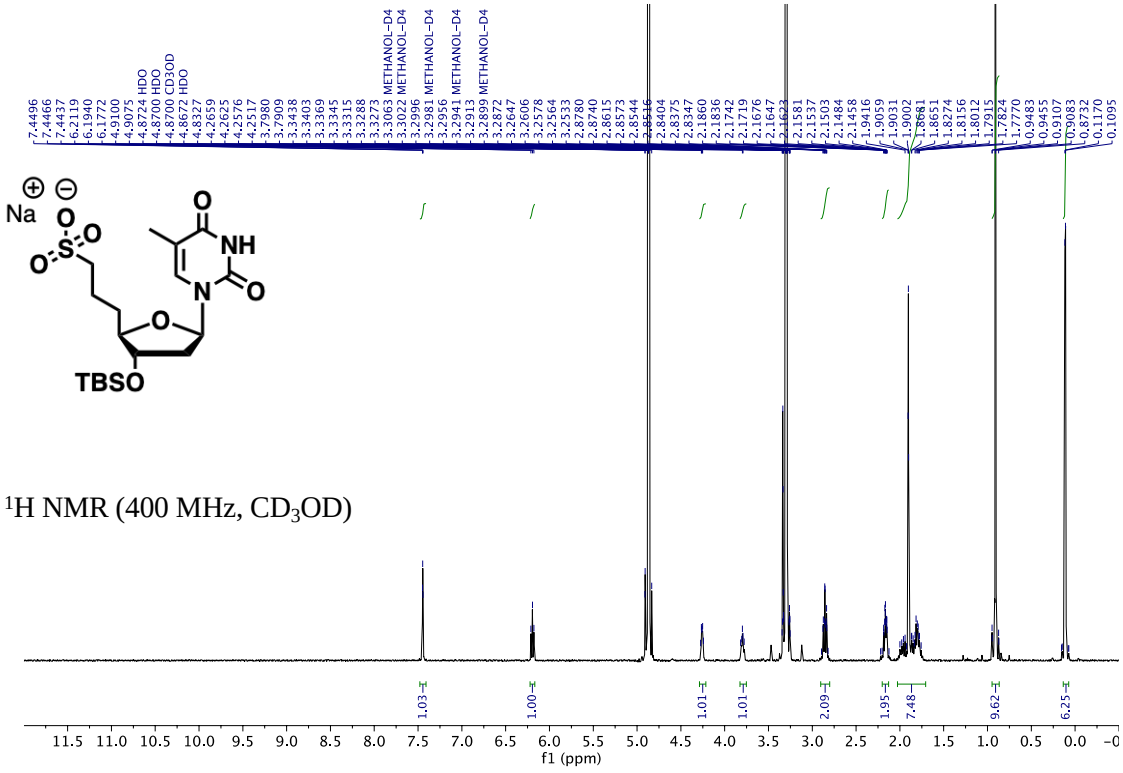
^{13}C NMR (101 MHz, $CDCl_3$)



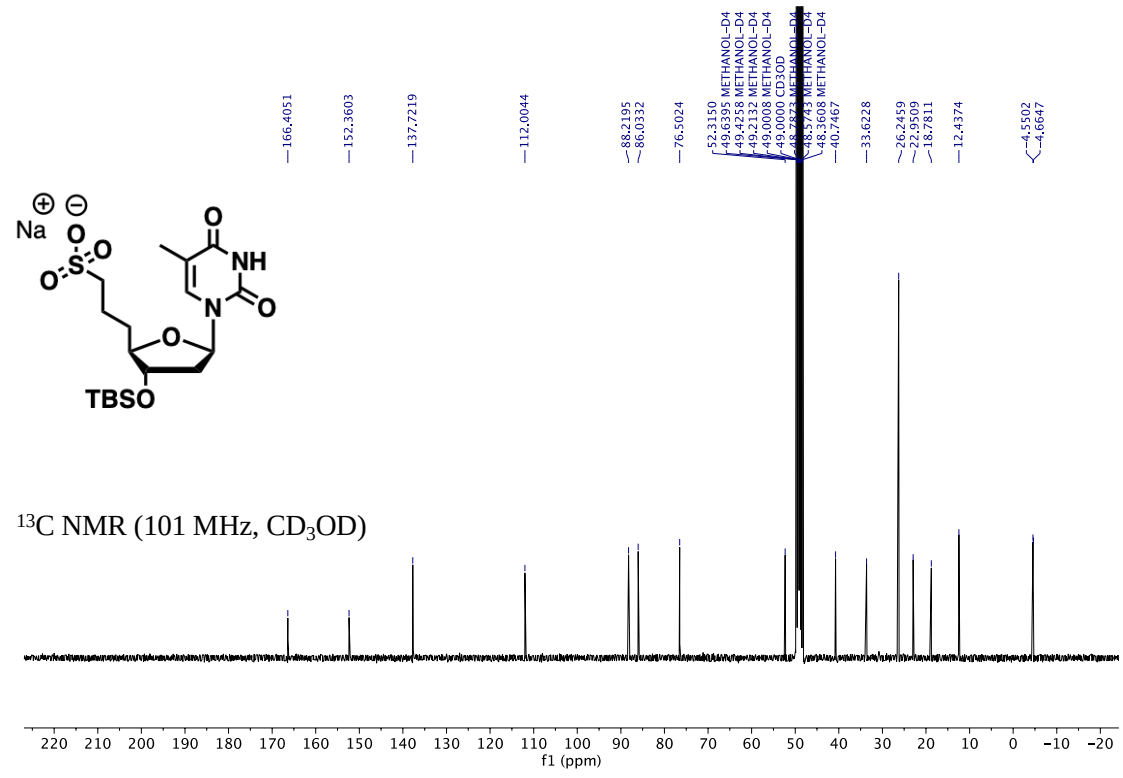
Compound 4

To a stirred solution of compound **3** (161 mg, 0.3 mmol) in acetone (3.2 mL) NaI (240 mg, 1.6 mmol) was added. The resulting reaction mixture was refluxed using an oil bath for 16 h. After cooled to room temperature, diol silica gel (2.5 g) was added to the solution, and was concentrated under reduced pressure. The dried diol silica gel was charged on diol silica gel column (20 g), and desired compound was purified using a gradient from 10 to 30% MeOH in EtOAc. The fractions containing compound **4** were collected and the solvents were removed by evaporation. As a result, the desired product **4** was obtained as colorless amorphous in quantitative yield (150 mg, 0.3 mmol).

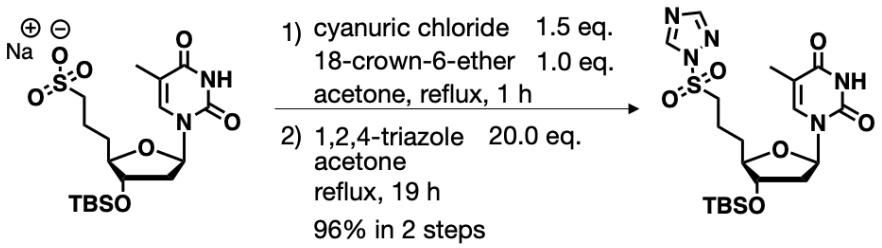
^{1}H NMR (400 MHz, CD_3OD) δ 7.45 (t, *J* = 1.2 Hz, 1H), 6.19 (t, *J* = 6.9 Hz, 1H), 4.26 (dt, *J* = 6.0, 3.5 Hz, 1H), 3.83 - 3.75 (m, 1H), 2.90 - 2.80 (m, 2H), 2.20 - 2.13 (m, 2H), 2.03 - 1.71 (m, 7H), 0.91 (d, *J* = 1.0 Hz, 9H), 0.11 (d, *J* = 3.0 Hz, 6H). Exchangeable protons were not observed (1H).
^{13}C{^{1}H} NMR (101 MHz, CD_3OD) δ 166.4, 152.4, 137.7, 112.0, 88.2, 86.0, 76.5, 52.3, 40.8, 33.6, 26.3, 23.0, 18.8, 12.4, -4.6, -4.7.
HRMS: m/z (ESI) calcd. for $C_{18}H_{31}N_2O_7SSi^-$ $[M]^-$ 447.1627, found 447.1615.



^{1}H NMR (400 MHz, CD_3OD)



^{13}C NMR (101 MHz, CD_3OD)



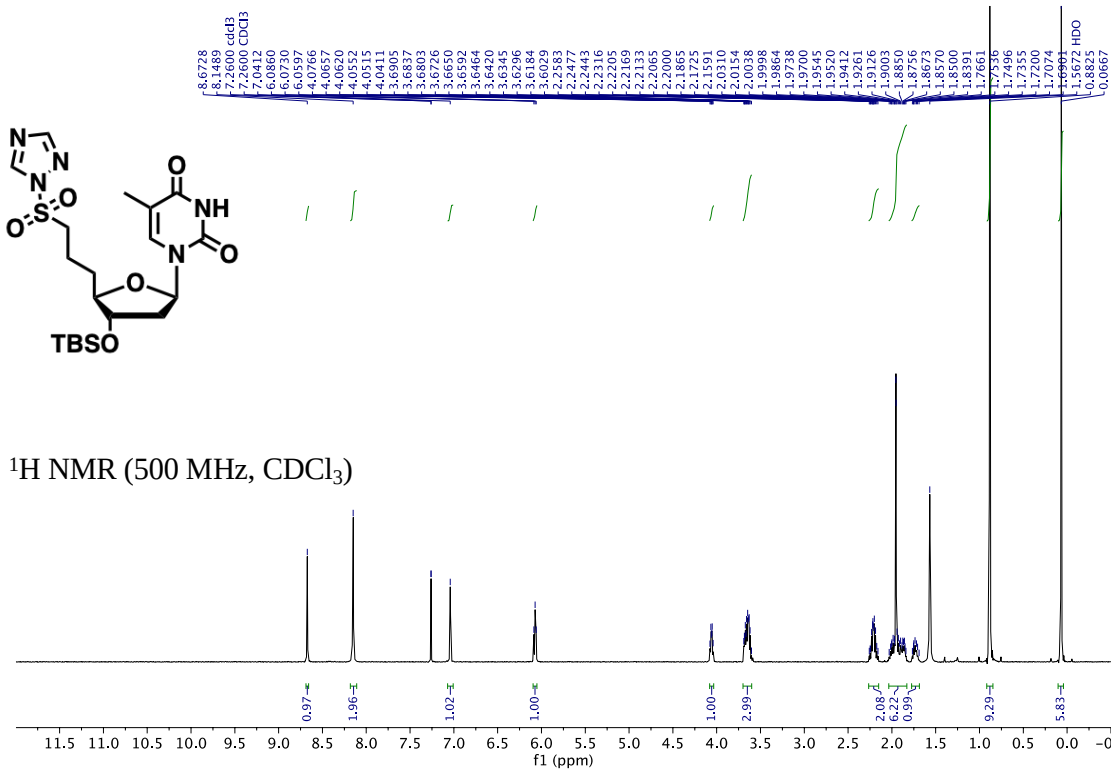
Compound 5

Compound **4** (1.10 g, 2.3 mmol) with 18-crown-6-ether (619 mg, 2.3 mmol) was rendered anhydrous by co-evaporation with dry acetone four times. To a stirred solution of the compound in acetone (23 mL) cyanuric chloride (647 mg, 3.5 mmol) was added. The resulting reaction mixture was refluxed using an oil bath for 1 h and then cooled down to room temperature. Then, to the stirring suspension was added 1,2,4-triazole (3.23 g, 46.8 mmol). The resulting reaction mixture was refluxed for 19 h and then cooled down to room temperature. After being filtered by using Celite, the mixture was concentrated *in vacuo* to give crude, which was purified using flash chromatography on silica gel using a gradient from 50 to 80% EtOAc in *n*-hexane and gave compound **5** as colorless syrup in 96% yield (1.12 g, 2.24 mmol).

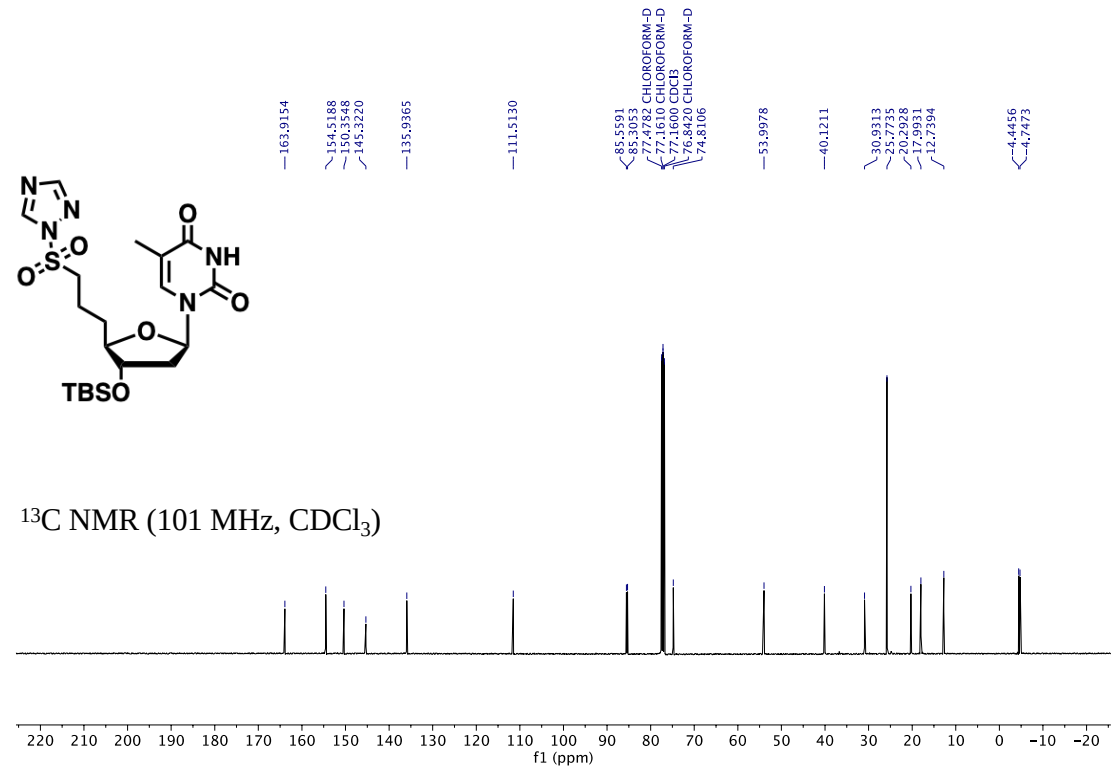
^{1}H NMR (500 MHz, $CDCl_3$) δ 8.67 (s, 1H), 8.15 (s, 2H), 7.04 (s, 1H), 6.07 (t, J = 6.6 Hz, 1H), 4.06 (dt, J = 7.1, 5.3 Hz, 1H), 3.70-3.60 (m, 3H), 2.26-2.15 (m, 2H), 2.04-1.83 (m, 6H), 1.77-1.69 (m, 1H), 0.88 (s, 9H), 0.07 (s, 6H).

^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ 163.9, 154.5, 150.4, 145.3, 135.9, 111.5, 85.6, 85.3, 74.8, 54.0, 40.1, 30.9, 25.8, 20.3, 18.0, 12.7, -4.4, -4.7.

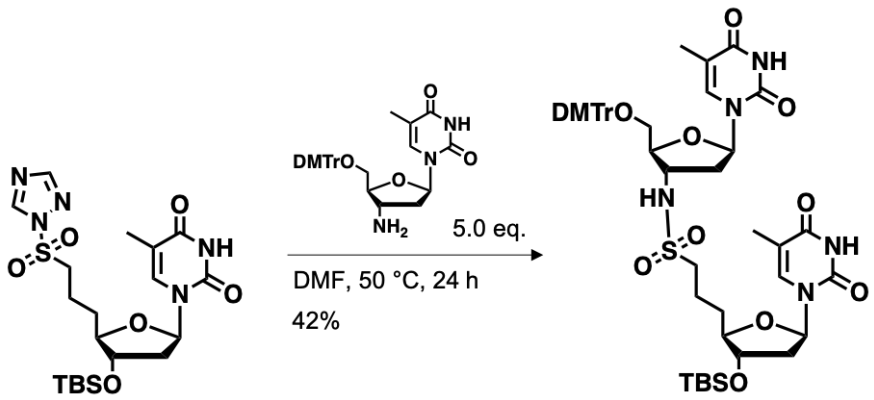
HRMS: m/z (ESI) calcd. for $C_{20}H_{33}N_5NaO_6SSi^+$ $[M + Na]^+$ 522.1813, found 522.1818.



^{1}H NMR (500 MHz, $CDCl_3$)



^{13}C NMR (101 MHz, $CDCl_3$)



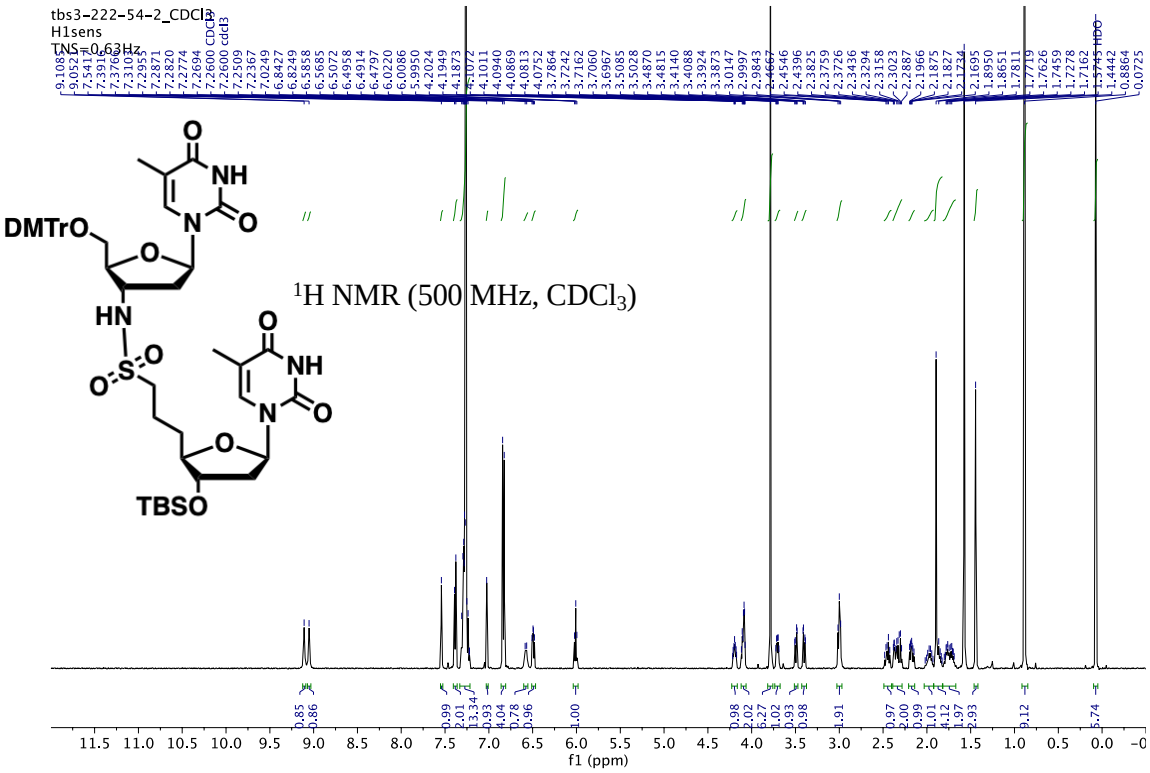
Compound 6

To a stirred solution of compound **5** (38 mg, 0.075 mmol) in DMF (75 µL) 3' - deoxy-3'-amino-5'-(4,4-dimethoxytrityl)thymidine (82 mg, 0.15 mmol) was added. The resulting reaction mixture was stirred at 50 °C using an oil bath for 24 h then cooled down to room temperature, after which was diluted with CH_2Cl_2 (20 mL). The solution was washed twice with H_2O (10 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated *in vacuo* to give crude, which was purified using flash chromatography on silica gel using a gradient from 20 to 100% EtOAc in *n*-hexane and gave the desired product **6** as colorless solid in 42% yield (31 mg, 0.032 mmol).

^{1}H NMR (500 MHz, $CDCl_3$) δ 9.11 (s, 1H), 9.05 (s, 1H), 7.54 (s, 1H), 7.38 (d, *J* = 7.5 Hz, 2H), 7.31-7.22 (m, 7H), 7.02 (s, 1H), 6.83 (d, *J* = 8.9 Hz, 4H), 6.58 (d, *J* = 8.7 Hz, 1H), 6.49 (dd, *J* = 8.0, 5.8 Hz, 1H), 6.01 (t, *J* = 6.8 Hz, 1H), 4.21-4.17 (m, 1H), 4.12-4.08 (m, 2H), 3.79 (s, 6H), 3.71 (dt, *J* = 8.6, 3.9 Hz, 1H), 3.49 (dd, *J* = 10.7, 2.8 Hz, 1H), 3.40 (dd, *J* = 10.8, 2.6 Hz, 1H), 3.00 (t, *J* = 7.6 Hz, 2H), 2.48-2.42 (m, 1H), 2.38-2.29 (m, 2H), 2.20-2.15 (m, 1H), 2.02-1.93 (m, 1H), 1.90-1.83 (m, 4H), 1.80-1.69 (m, 2H), 1.44 (s, 3H), 0.89 (s, 9H), 0.07 (s, 6H).

^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ 164.0, 163.4, 158.9, 151.5, 150.4, 144.4, 136.7, 135.4, 135.3, 135.0, 130.2, 128.3, 128.2, 127.4, 113.4, 112.5, 111.3, 87.2, 86.3, 86.0, 84.3, 75.1, 63.5, 55.4, 54.5, 53.2, 39.7, 38.7, 31.6, 25.8, 20.9, 18.1, 12.7, 12.0, -4.5, -4.7.

HRMS: m/z (ESI) calcd. for $C_{49}H_{63}N_5NaO_{12}SSi^+$ $[M]^+$ 996.3855, found 996.3852.


tbs3-222-54-2_CDCl3
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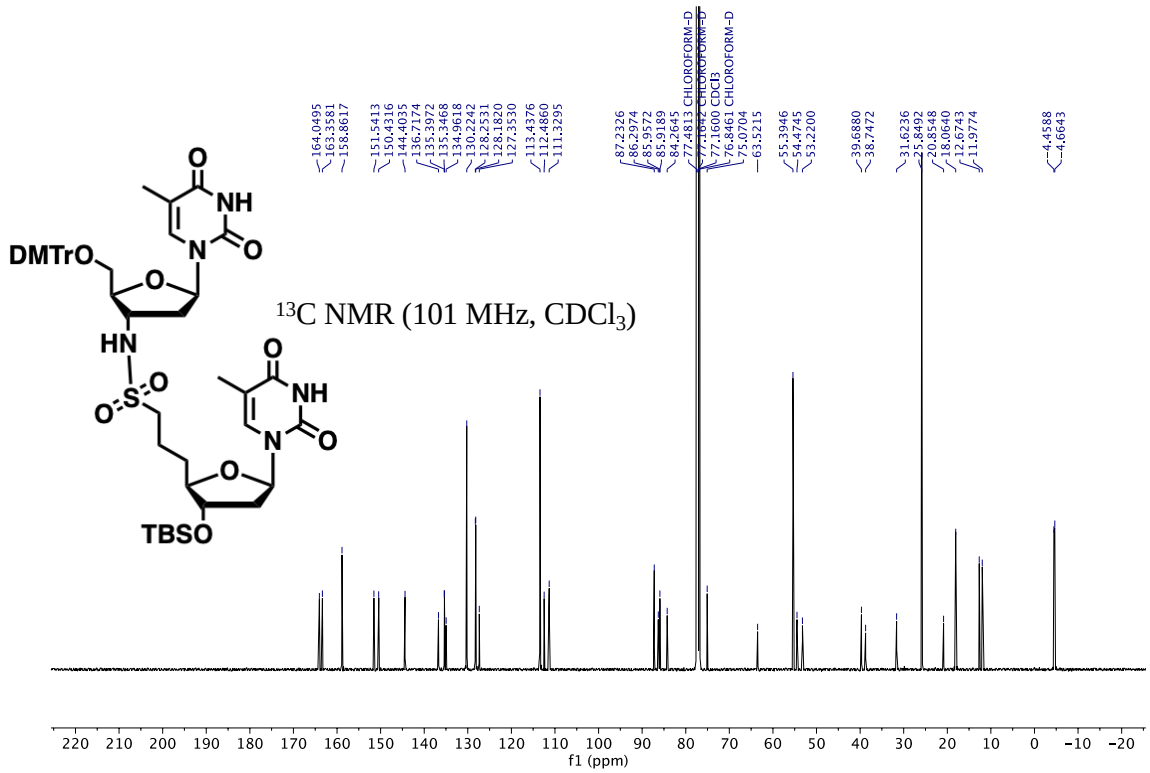
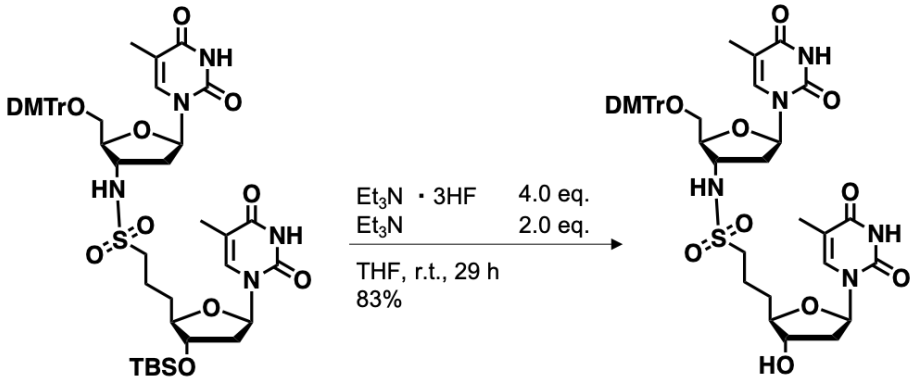
TNS=0.63Hz
DMTrO
NH
O
N
HN
S
TBSO
^{1}H NMR (500 MHz, $CDCl_3$)
f1 (ppm)


DMTrO
NH
O
N
HN
S
TBSO
^{13}C NMR (101 MHz, $CDCl_3$)
f1 (ppm)



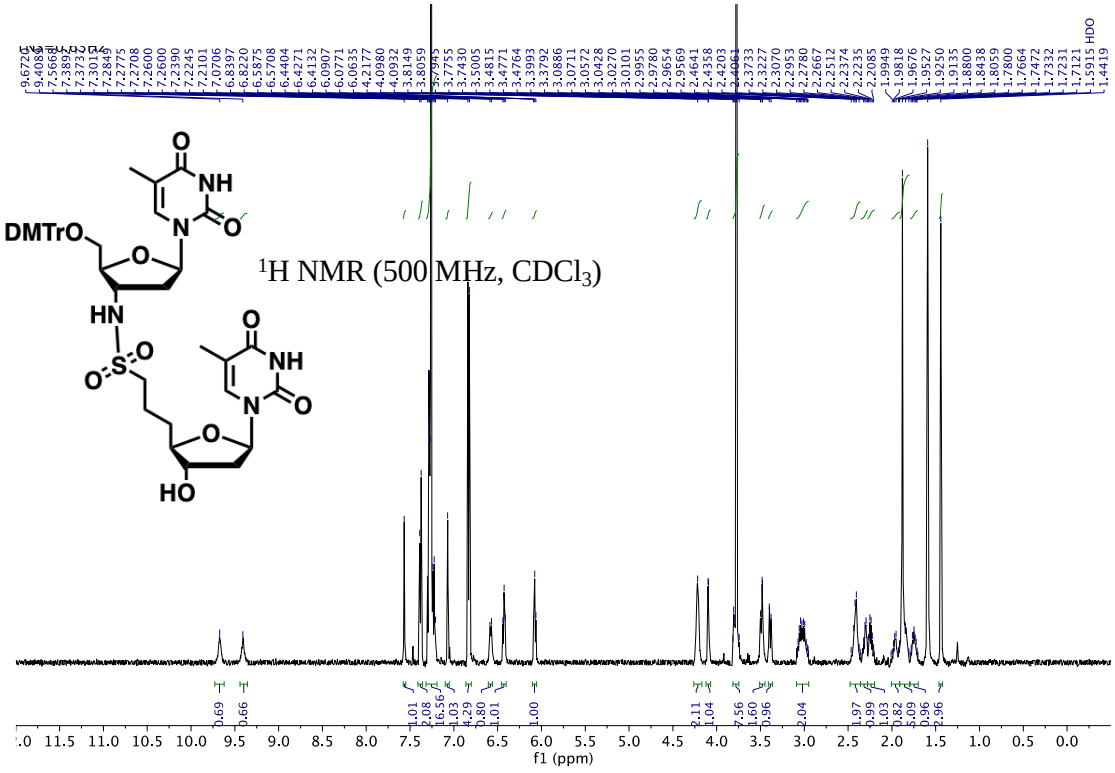
Compound 7

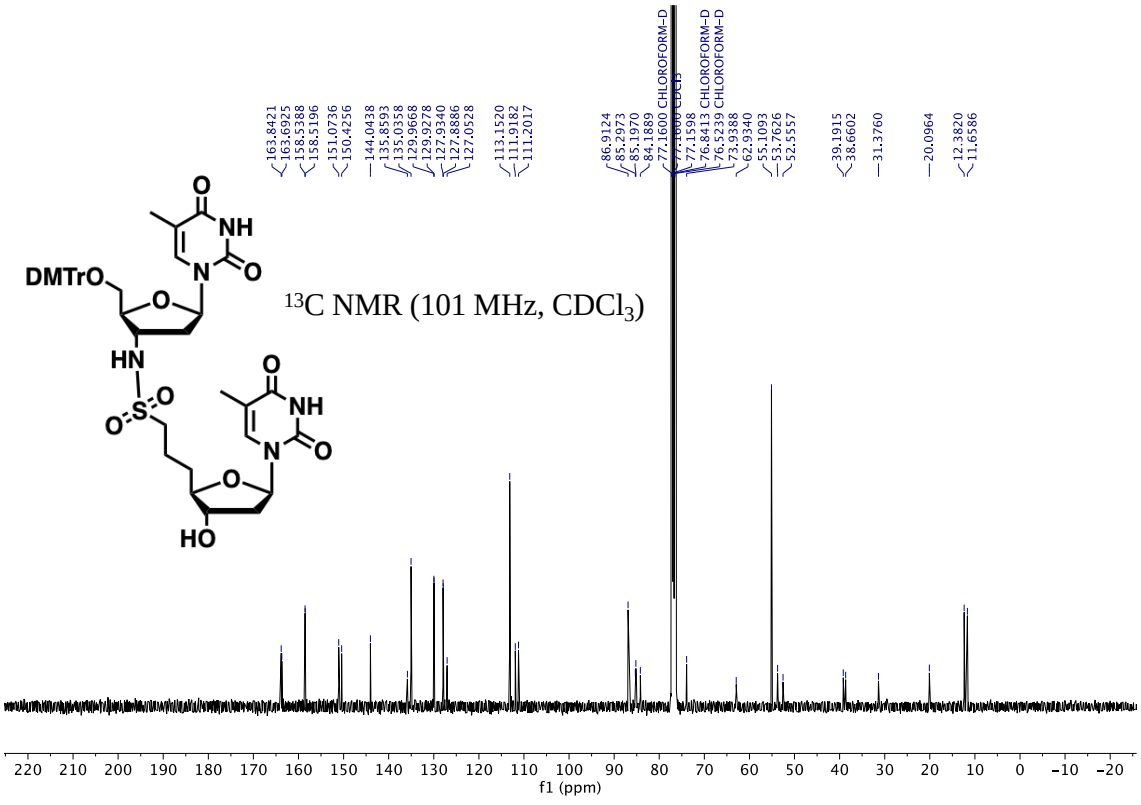
To a stirred solution of compound **6** (219 mg, 0.23 mmol) in THF (2.3 mL), Et_3N (62 μL, 0.45 mmol) and $Et_3N{\cdot}3HF$ (147 μL, 0.90 mmol) was added. The resulting reaction mixture was stirred at room temperature for 29 h, after which Et_3N (62 μL, 0.45 mmol) and $Et_3N{\cdot}3HF$ (147 μL, 0.90 mmol) was added. The reaction mixture was stirred at room temperature for another 24 h, after which was quenched with trimethylethoxysilane (700 μL). Silica gel (3 g) was added to the solution, and was concentrated under reduced pressure. The dried silica gel was charged on silica gel column (10 g), and desired compound was purified using a gradient from 0 to 20% MeOH in CH_2Cl_2. The fractions containing compound **7** were collected and the solvents were removed by evaporation. As a result, the desired product **7** was obtained as colorless solid in 83% yield (161 mg, 0.19 mmol).

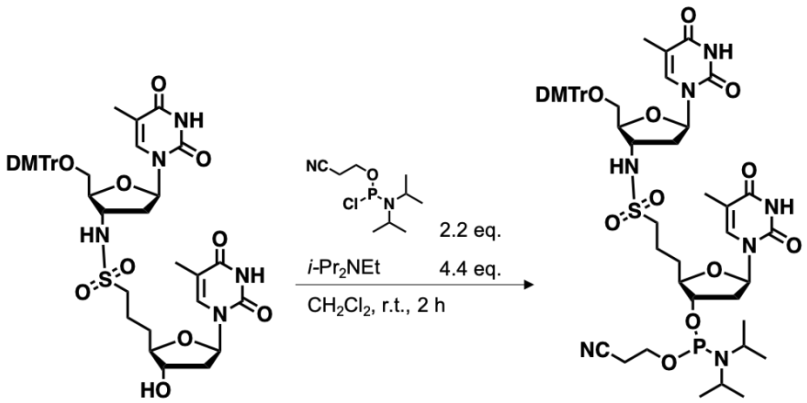
^{1}H NMR (500 MHz, $CDCl_3$) δ 9.67 (s, 1H), 9.41 (s, 1H), 7.57 (s, 1H), 7.38 (d, J = 7.8 Hz, 2H), 7.30-7.07 (m, 7H), 7.07 (s, 1H), 6.83 (d, J = 8.8 Hz, 4H), 6.58 (d, J = 8.4 Hz, 1H), 6.43 (t, J = 6.8 Hz, 1H), 6.08 (t, J = 6.8 Hz, 1H), 4.22 (s, 2H), 4.10 (d, J = 2.4 Hz, 1H), 3.81-3.74 (m, 8H), 3.51-3.45 (m, 2H), 3.39 (d, J = 10.0 Hz, 1H), 3.09-2.95 (m, 2H), 2.46-2.36 (m, 2H), 2.35-2.28 (m, 1H), 2.27-2.20 (m, 1H), 2.01-1.93 (m, 1H), 1.91-1.81 (m, 5H), 1.80-1.70 (m, 1H), 1.44 (s, 3H).

^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ 163.8, 163.7, 158.5, 151.1, 150.4, 144.0, 135.9, 135.0, 130.0, 129.9, 127.9, 127.1, 113.2, 111.9, 111.2, 86.9, 85.3, 85.2, 84.2, 73.9, 55.1, 53.8, 52.6, 39.2, 38.7, 31.4, 20.1, 12.4, 11.7.

HRMS: m/z (ESI) calcd. for $C_{43}H_{49}N_5NaO_{12}S^+$ $[M + Na]^+$ 882.2991, found 882.3008.


DMTrO
NH
O
N
HN
S
HO
^{1}H NMR (500 MHz, CDCl$_3$)
f1 (ppm)


DMTrO
NH
O
N
HN
S
HO
^{13}C NMR (101 MHz, CDCl$_3$)
f1 (ppm)

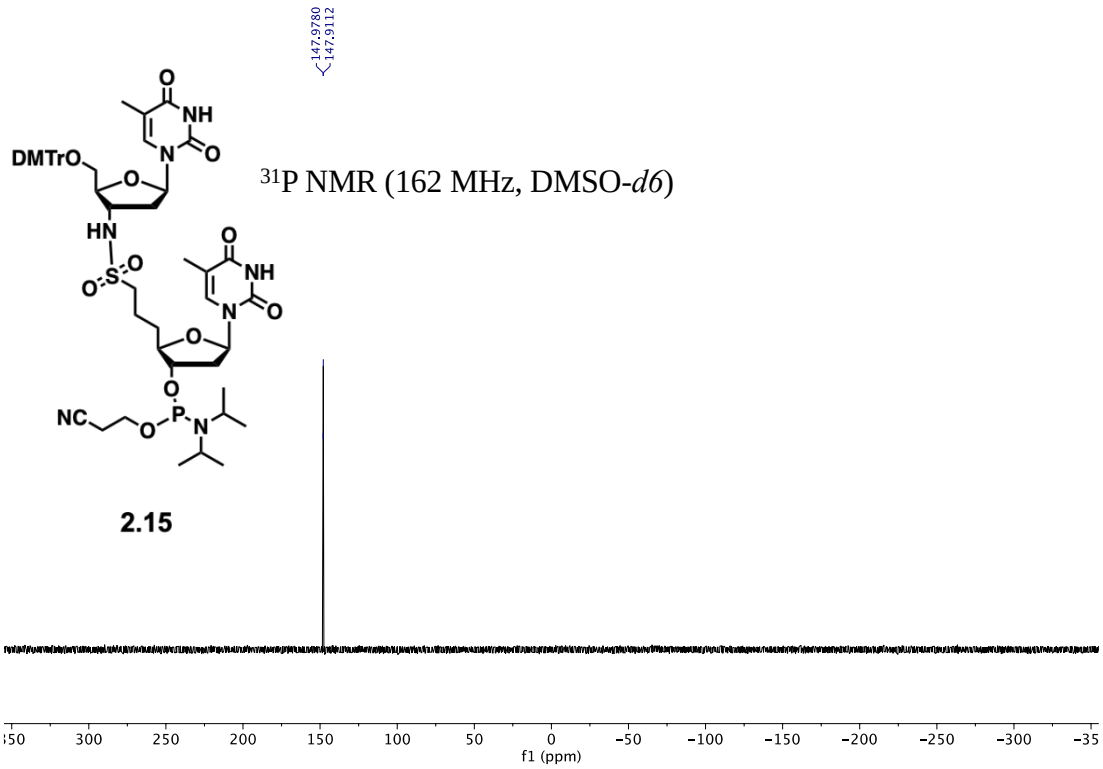


Compound 8

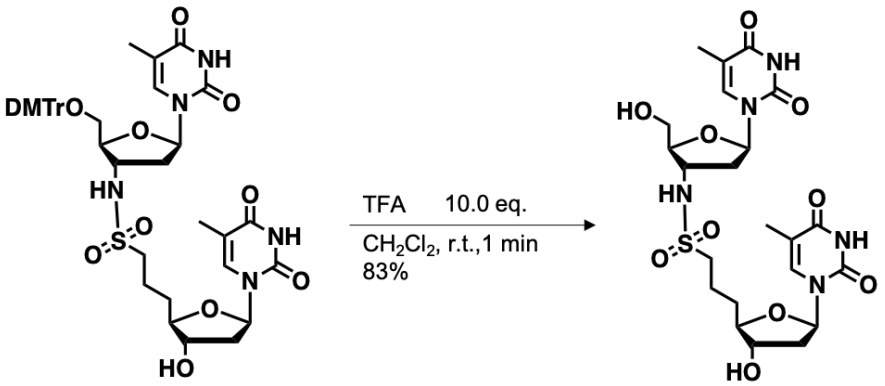
compound **7** was rendered anhydrous by co-evaporation with dry pyridine nine times, and toluene six times. To a stirred solution of compound **7** (100 mg, 0.12 mmol) in CH_2Cl_2 (1.2 mL) diisopropylethylamine (88.4 μL, 0.52 mmol), and 2-cyanoethyl *N*, *N*-diisopropylchlorophosphoramidite was added. The resulting reaction mixture was stirred at room temperature for 2 h. CH_2Cl_2 (50 mL) was added to the reaction mixture , and it was washed three times with saturated $NaHCO_3$ solution (30 mL) and once with saturated NaCl solution (30 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated *in vacuo* to give crude residue, which was purified using flash chromatography on NH silica gel using a gradient from 0 to 10% MeOH in CH_2Cl_2 and removed residue of decomposed *N*,*N*-diisopropylchlorophosphoramidite as the ^{31}P NMR chart below. The compound was obtained quantitively, and introduced into oligodeoxynucleotide without further purification.

^{31}P NMR (162 MHz, DMSO-d_6) δ 148.0, 147.9

HRMS: m/z (ESI) calcd. for $C_{52}H_{66}N_7NaO_{13}PS+$ $[M + Na]^+$ 1082.4069, found 1082.4062.



^{31}P NMR (162 MHz, DMSO-*d6*)



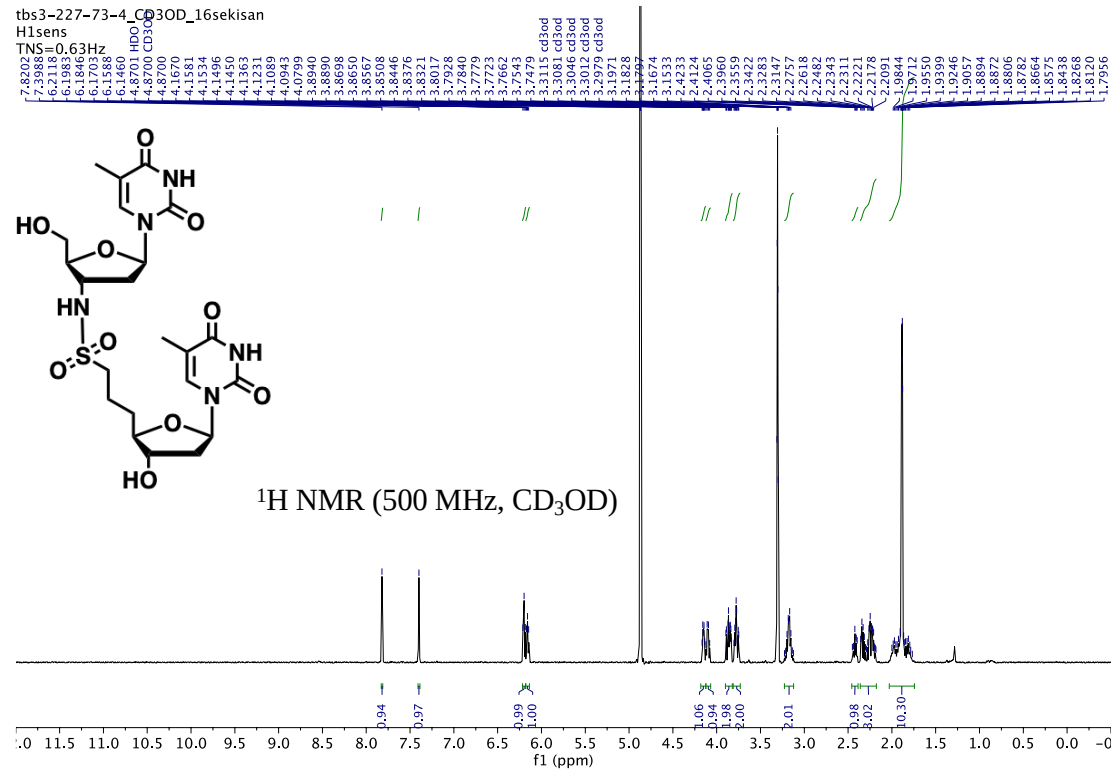
TnsccT

To a stirred solution of compound **7** (12.0 mg, 0.014 mmol) in CH_2Cl_2 (209 μL) trifluoroacetic acid (11 μL, 0.14 mmol) was added. The resulting reaction mixture was stirred at room temperature for 1 min, after which was quenched with diethylaminomethyl-polystyrol (53.1 mg, 0.17 mmol) in CH_2Cl_2-MeOH (1 mL, 1 mL) at 0 °C for 1.5 min. Then, diethylaminomethyl - polystyrol was removed by filtration. The filtrate was concentrated *in vacuo* to give crude, which was purified using flash chromatography on silica gel using a gradient from 0% to 30% MeOH in CH_2Cl_2 and gave the desired product **9** as colorless solid in 83% yield (6.5 mg, 0.12 mmol).

^{1}H NMR (500 MHz, CD_3OD) δ 7.82 (s, 1H), 7.40 (s, 1H), 6.20 (t, J = 6.8 Hz, 1H), 6.16 (t, J = 6.1 Hz, 1H), 4.17-4.14 (m, 1H), 4.10 (q, J = 7.2 Hz, 1H), 3.90-3.83 (m, 2H), 3.80-3.75 (m, 2H), 3.23-3.12 (m, 2H), 2.45-2.40 (m, 1H), 2.36-2.18 (m, 3H), 2.00-1.77 (m, 10H). Exchangeable protons were not observed (5H).

^{13}C{^{1}H} NMR (101 MHz, CD_3OD) δ 166.5, 166.4, 152.3, 138.2, 137.8, 112.0, 111.4, 87.0, 86.6, 86.0, 85.8, 75.1, 61.4, 53.4, 53.0, 40.0, 39.9, 32.9, 21.9, 12.5.

HRMS: m/z (ESI) calcd. for $C_{22}H_{31}N_5NaO_{10}S^+$ $[M + Na]^+$ 580.1684, found 580.1694.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TNS=0.63Hz
^{1}H NMR (500 MHz, CD_3OD)
f1 (ppm)

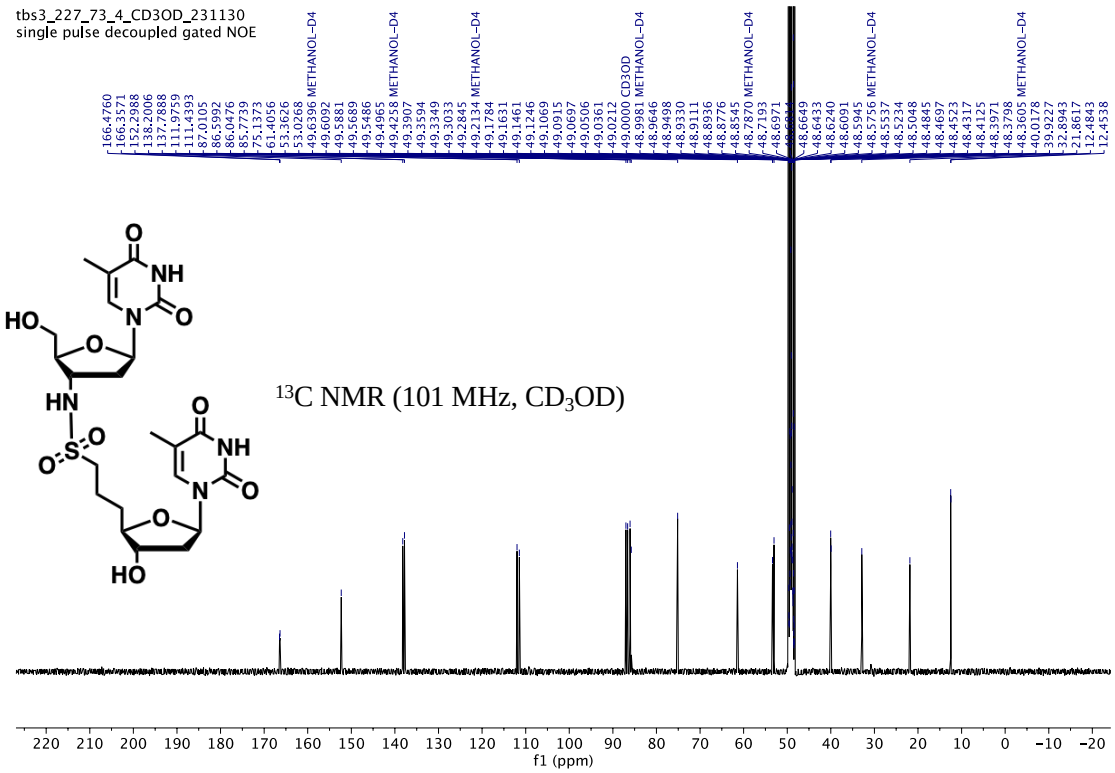

tbs3_227_73_4_CD3OD_231130
single pulse decoupled gated NOE
^{13}C NMR (101 MHz, CD_3OD)
f1 (ppm)

Figure S1. Representative melting curves of **ODN1-9** and **ASO1-3**

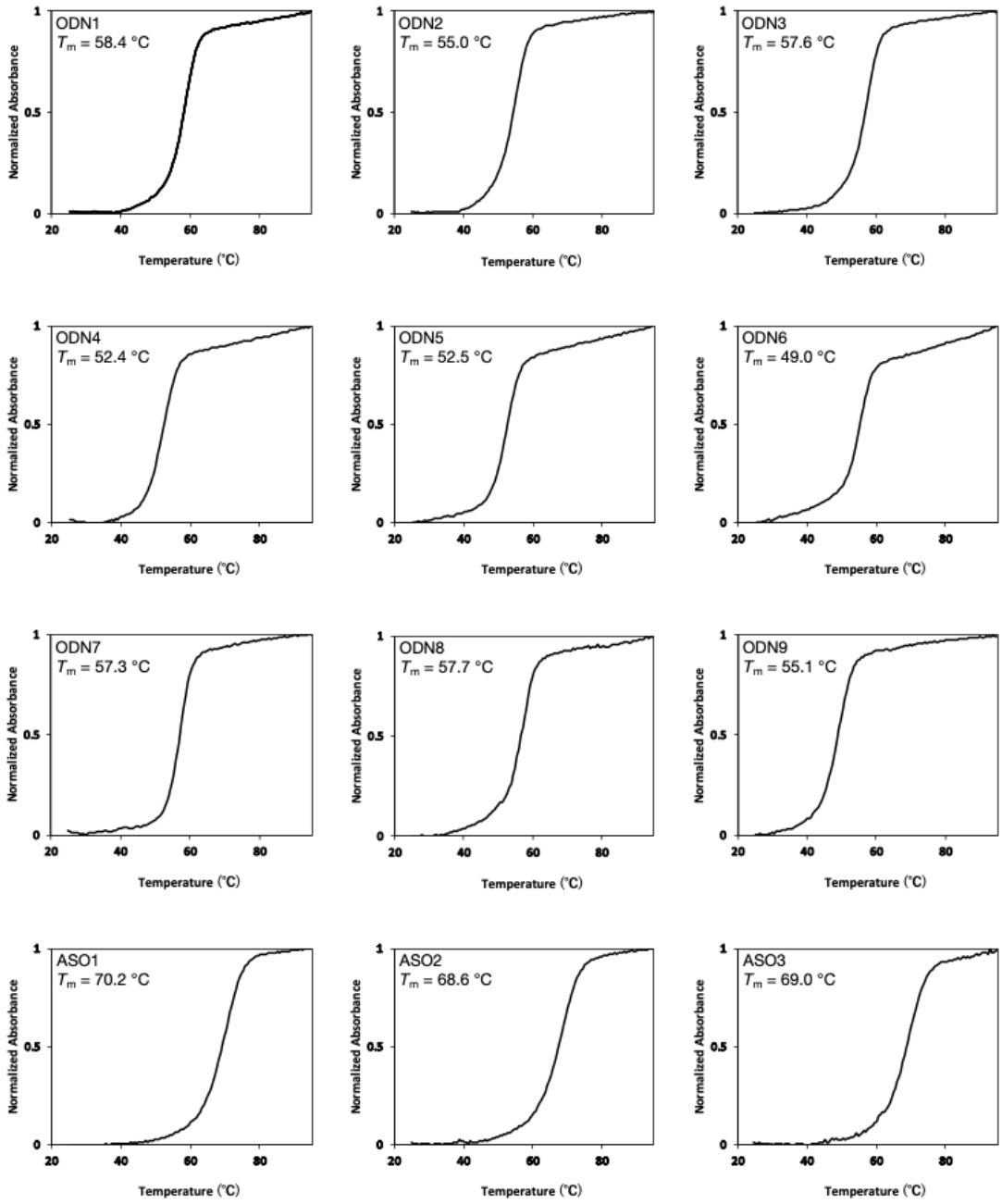
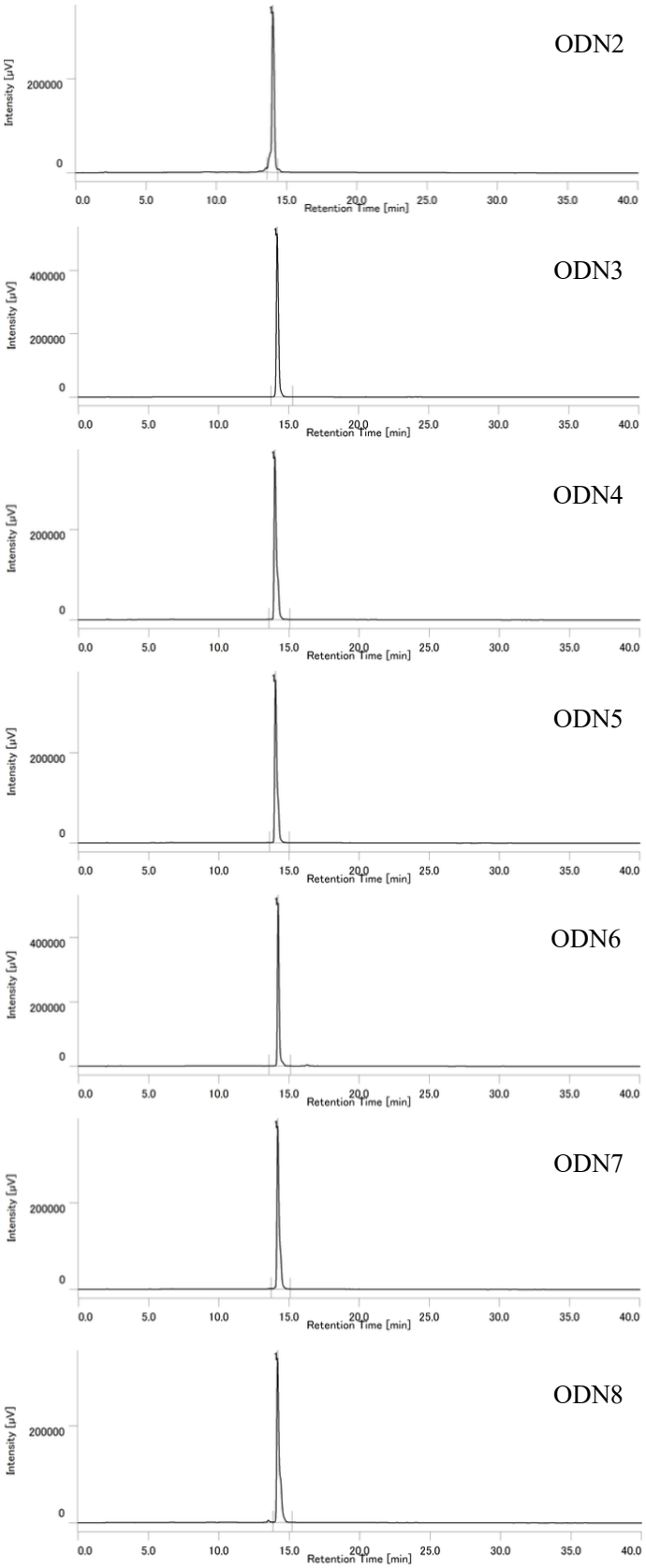
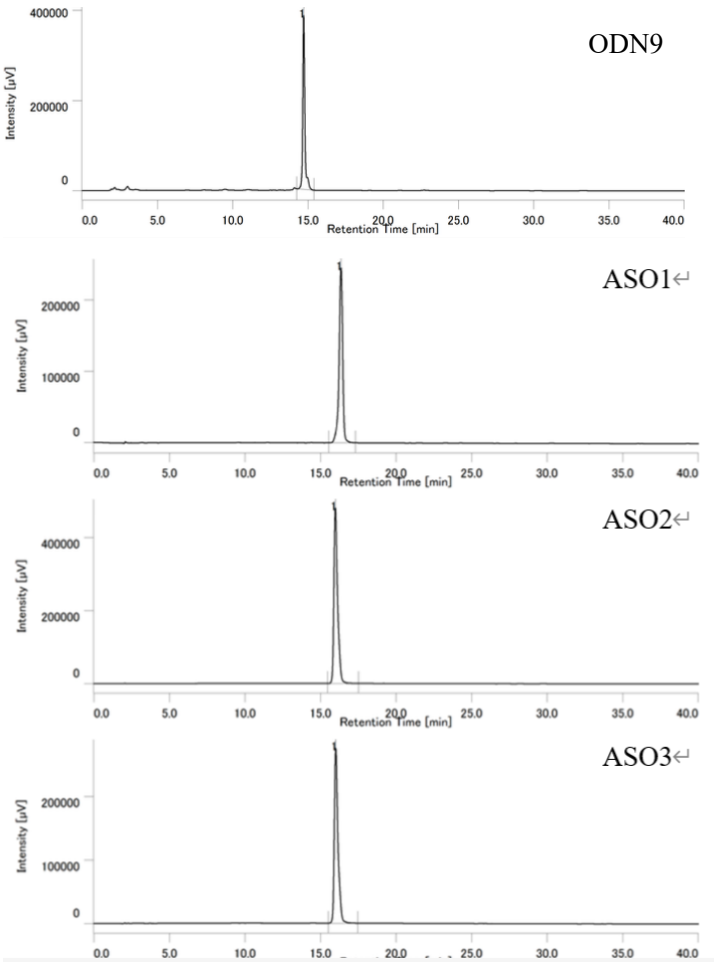


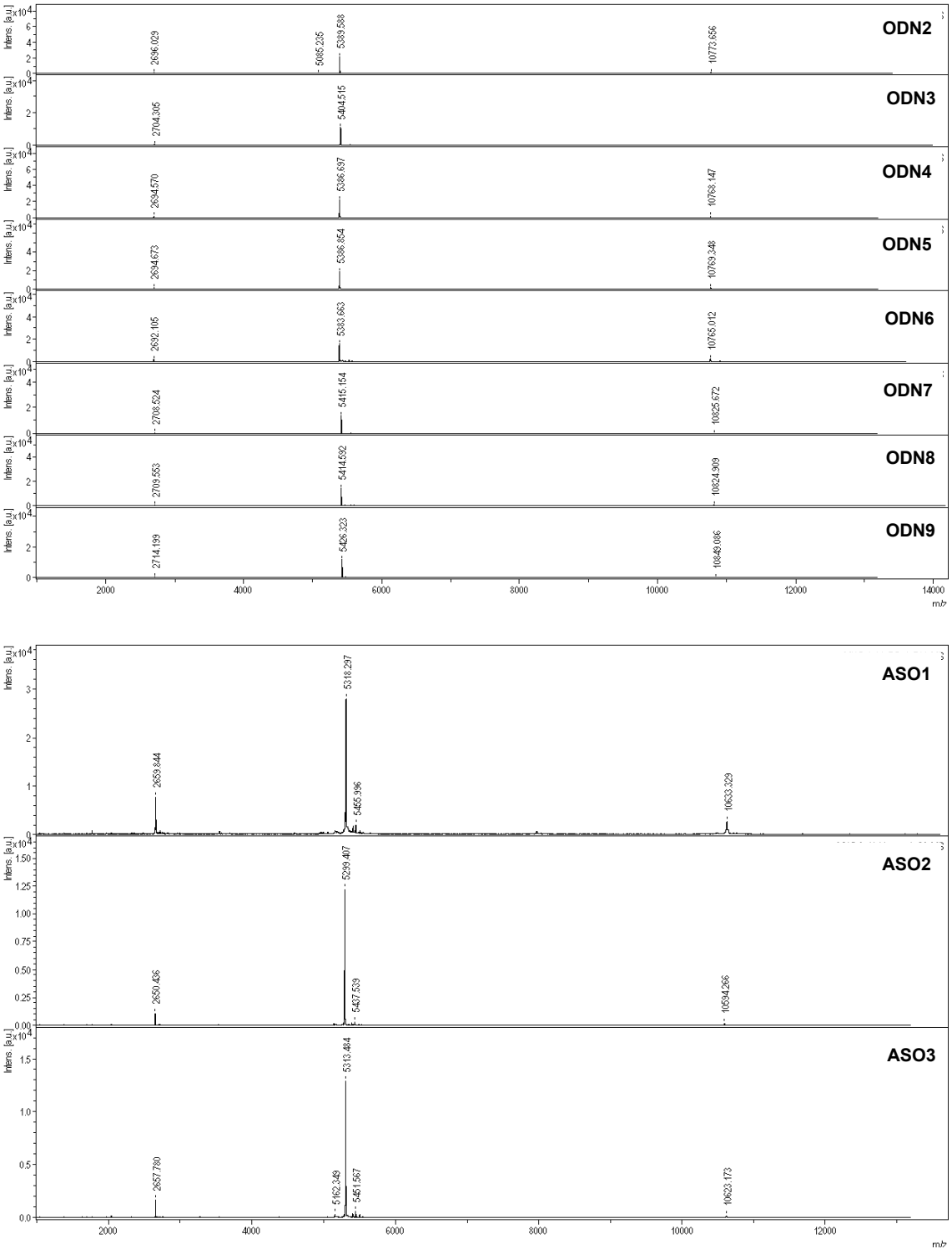
Figure S2. HPLC charts of purified each ODN and ASO





Conditions: A solution: 8 mM Et_3N, 100 mM HFIP buffer, B solution: MeOH. Column: C18 reversedphase column, 4.6 mm x150 mm, flow rate: 1 mL/min. Gradient: 5% B solution to 45% B solution in 0 min-40 min at 65 °C. The peaks were detected at 254 nm.

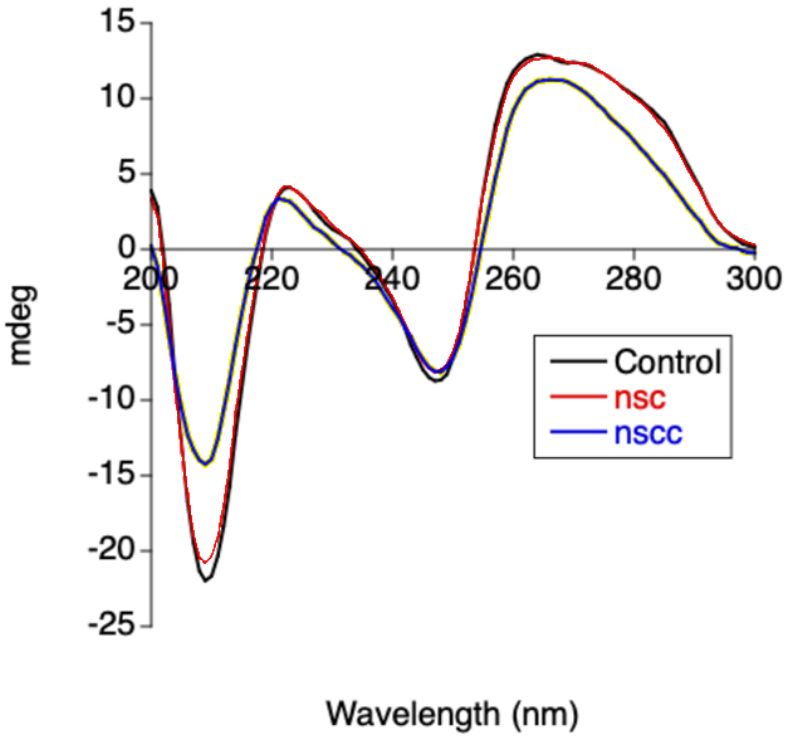
Figure S3. MALDI-Mass spectrum of purified each ODN and ASO



Name	Sequence (5’ to 3’)	Yield(%)	calcd. $[M+H]^+$	Found $[M+H]^+$
ODN2	CGCACTTTxxTTTCGACC	18	5389.7	5389.6
ODN3	CGCACTTTyyTTTCGACC	15	5403.7	5404.5
ODN4	CGCACT[xx][xx]TTTCGACC	9	5386.8	5386.7
ODN5	CGCACTTT[xx][xx]TCGACC	14	5386.8	5386.9
ODN6	CGCACT[xx][xx][xx]TCGACC	10	5383.9	5384.6
ODN7	CGCACT[yy][yy]TTTCGACC	15	5413.8	5415.2
ODN8	CGCACTTT[yy][yy]TCGACC	10	5413.8	5414.6
ODN9	CGCACT[yy][yy][yy]TCGACC	10	5425.0	5426.3
ASO2	AccGAGGcxxGcATAc	6	5299.4	5299.4
ASO3	AccGAGGcyyGCATAc	8	5313.4	5313.5

ODN1 and ASO1 were purchased from GeneDesign, Inc.

Figure S4. CD spectra of ODNs with the complementary RNA



4.0 μM duplex in 10 mM sodium phosphate, 100 mM sodium chloride, 0.1 mM EDTA, pH 7.0.
control: **ODN1**, nsc: **ODN2**, nscc: **ODN3**

Molecular modeling Thymidyl(3′,5′)thymidine was extracted from an A-type duplex generated using the Nucleic Acid Builder (NAB) in AmberTools, and hydrogen atoms were added to the 5′ and 3′ termini. The phosphodiester linkage of the dimer was then manually converted to the nscc linkage.
Conformational searches were subsequently performed using RDKit. The positions of all heavy atoms of the 5′-residue, as well as the thymine moiety and the C1′, C2′, C3′, and C4′ atoms of the 3′-residue, were fixed. The S–C bond was cleaved, and the C2′–C3′–N–S dihedral angle of the 5′-residue, together with the C3′–C4′–C5′–C6′ and C4′–C5′–C6′–C7′ dihedral angles of the 3′-residue, were sampled at every staggered conformation.
Conformations in which the distance between the sulfur atom and the C7′ atom was within 2.0 Å were selected, after which the S–C bond was reformed. The resulting structures were then subjected to geometry optimization using the UFF force field, yielding two representative structures. The structures were visualized using Maestro 14.5.